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METHOD OF ASSESSING SKIN

FIELD OF THE INVENTION

The present invention relates to a method of assessing skin using fluorescence.

BACKGROUND OF THE INVENTION

The native fluorescence of human and mouse skin has been shown to vary with aging and UV exposure in a predictable manner. See Brancaleon et al., J. Invest. Dermatol. 113(6):977-982, 1999; Kollias et al., J. Invest. Dermatol.111(5):776-780, 1998; Leffell et al., Arch Dermatol. 124(10):1514-1518, 1988; Na et al.,J. Invest. Dermatol. 116(4):536-540, 2001; and Tian et al., J. Invest. Dermatol. 116(6):840-845, 2001. Thus, fluorescence spectroscopy has been proven to be an objective quantitative method for studying skin aging and photoaging.

The major fluorescence bands that have been detected by *in vivo* fluorescence spectroscopy include: a) a band assigned to tryptophan (maximum at 295 nm excitation, 345 nm emission), b) a band assigned to pepsin digestible collagen cross-links (335 nm excitation, 390 nm emission), c) a band assigned to collagenase digestible collagen cross-links (370 nm excitation, 460 nm emission), and d) a band most likely due to elastin and collagen cross-links (390-420 nm broad band excitation, 500 nm emission). See Gillies et al. J. Invest. Dermatol, 115(4):704-707, 2000. Secondary fluorescence bands have been identified that may be related to collagen peroxidation (Odetti et al.Lab Invest. 70(1):61-67, 1994) or elastin (Leffell et al., Arch Dermatol., 124(10):1514-1518, 1988): one at 356 nm

excitation, 420 nm emission and another at 390 nm excitation, 460 nm emission respectively.

The fluorescence signal assigned to tryptophan moieties measured *in situ* was found to increase when epidermal proliferation increases. See Kollias et al., J. Invest. Dermatol, 111(5):776-780,1998 and Zhang et al., Lasers Surg. Med. 20(3):319-331, 1997. This was verified by inducing epidermal repair after mechanical insult, e.g. tape stripping. See Brancaleon et al., J. Invest. Dermatol. 113(6):977-982, 1999. Furthermore, α-hydroxy-acid-induced increases of cellular turnover in human epidermis caused the 295 nm excitation band to increase in a dose dependent manner. See Doukas et al., Photochem. Photobiol. 74(1):96-102, 2001. In SKH hairless mice the fluorescence due to tryptophan moieties decreases with age, implying an age-related reduction of the epidermal cell turnover rate. See Kollias et al., J. Invest. Dermatol. 111(5):776-780, 1998.

Non-enzymatic glycosilation of proteins occurs spontaneously with aging (See Monnier et al., Clin Endocrinol Metab 11(2):431-452, 1982; Njoroge et al., J. Biol. Chem. 263(22):10646-10652, 1988; Sell et al., J. Biol Chem 264(36):21597-21602, 1989; and Shaklai et al., J. Biol Chem 259(6):3812-3817, 1984) resulting in increased protein absorbance and fluorescence (Maillard reaction). The glucose-protein adduct rearranges and dehydrates to form brown and fluorescent pigments, which may form cross-links resulting in decreased protein solubility and altered mechanical properties. Such cross-links are evident in long-lived proteins, such as elastin and collagen. The accumulation of fluorescing cross-links in collagen has been used as a marker for the observed accelerated rate of aging in diabetes. See Monnier et al., Clin. Endocrinol.

Metab 11(2):431-452, 1982. In SKH mice the magnitude of the pepsin digestible collagen cross-link fluorescence maximum increases with chronological aging, whereas the increase in the magnitude of the collagenase digestible collagen cross-link and the elastin-associated fluorescence maxima is modest. See Kollias et al., J. Invest. Dermatol. 111(5):776-780, 1998. Similar trends have been observed in rats *ex vivo* (Odetti et al., Lab Invest. 70(1):61-67, 1994), in human buttock skin *in vivo* (Na et al., J. Invest. Dermatol 116(4):536-540, 2001), and in *ex vivo* human dermis taken from skin around the operating area of patients undergoing vascular surgery (Odetti et al., Metabolism 41(6)655-658, 1992).

Applicants have surprisingly found that skin native autofluorescence is a tool to evaluate skin health and the effects of aging (e.g., chronological aging as well as photoaging) on skin health.

SUMMARY OF THE INVENTION

In one aspect, the present invention features a method of determining skin health of an area of skin by (i) exposing the area of skin to a first exposure radiation to induce the area of skin to emit a first fluorescent emission, wherein the first exposure radiation comprises primarily of wavelengths of from about 290 nm to about 300 nm; (ii) measuring the intensity of the first fluorescent emission having a wavelength of from about 320 to about 350; (iii) exposing the area of skin to a second exposure radiation to induce the area of skin to emit a second fluorescent emission, wherein the second exposure radiation comprises primarily of wavelengths of from about 330-420 nm; (iv) measuring the intensity of the second fluorescent

emission having a wavelength of from about 380-470; (v) calculating a ratio of the intensity measured in step (ii) to the intensity measured in step (iv); and (vi) comparing the ratio to a control ratio.

In another aspect, the present invention features a method of determining the effect of a treatment to the skin of a subject by: (i) exposing a first area of skin to a first exposure radiation to induce the area of skin to emit a first fluorescent emission, wherein the first exposure radiation comprises primarily of wavelengths of from about 290 nm to about 300 nm and wherein the first area of skin was exposed to the composition; (ii) measuring the intensity of the first fluorescent emission having a wavelength of from about 320 to about 350;(iii) exposing the first area of skin to a second exposure radiation to induce the area of skin to emit a second fluorescent emission, wherein the second exposure radiation comprises primarily of wavelengths of from about 330-420 nm; (iv) measuring the intensity of the second fluorescent emission having a wavelength of from about 380-470; (v) calculating a ratio of the intensity measured in step (ii) to the intensity measured in step (iv);(iv) repeating steps (i) to (v) for a second area of skin, wherein the second area of skin was not exposed to the composition; and (vii) comparing the ratio for the first area of skin to the ratio for the second area of skin.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the excitation spectra of two individuals of 30 (dotted lines) and 60 (solid lines) years of age, respectively.

FIG. 2a is a graph showing the age distribution of the fluorescence intensity for the 295 nm excitation band.

FIG. 2b is a graph showing the age distribution of the fluorescence intensity for the 335 nm excitation band.

FIG. 2c is a graph showing the age distribution of the fluorescence intensity for the 360 nm excitation band.

FIG. 2d is a graph showing the age distribution of the fluorescence intensity for the 390 nm excitation band.

FIG. 3 is a graph showing the age distribution of the florescence intensity for 295 nm excitation band normalized to the fluorescence intensity of the 390 nm excitation band.

FIG. 4a is a graph showing the age distribution of the normalized florescence intensity for a sun-exposed area of the skin.

FIG. 4b is a graph showing the age distribution of the normalized florescence intensity for a sun-protected area of the skin.

FIG. 5a is a graph showing the change in fluorescence intensity over time at 295 nm excitation for retinol (active) and placebo treated skin.

FIG. 5b is a graph showing the change in fluorescence intensity over time at 390 nm excitation for retinol (active) and placebo treated skin.

FIG. 5c is a graph showing normalized florescence intensity over time for for retinol (active) and placebo treated skin.

DETAILED DESCRIPTION OF THE INVENTION

It is believed that one skilled in the art can, based upon the description herein, utilize the present invention to its fullest extent. The following specific embodiments are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Also, all publications, patent applications, patents, and other references mentioned herein are incorporated by reference. Unless otherwise indicated, a percentage refers to a percentage by weight (i.e., %(W/W)).

Exposure Radiation

In one embodiment, the area(s) of skin are exposed to at least two exposure radiations (e.g., from UV radiation sources such as xenon arc lamps or mercury lamps). In one embodiment, the first exposure radiation includes primarily wavelengths of from about 290 nm to about 300 nm and the second exposure radiation includes primarily wavelengths of from about 330-420 nm. What is meant by "primarily" is at

least half of the wavelengths of the exposure radiation. In a further embodiment, the first exposure radiation includes primarily wavelengths of about 295 nm and the second exposure radiation includes primarily wavelengths of from about 390 to about 410 nm.

The exposure radiations are directed to the skin in order emit a fluorescent emission and to measure the intensity of such emission (e.g., a specific wavelength or wavelength range). In one embodiment, the method includes measuring the intensity of the first fluorescent emission having a wavelength of from about 330 nm to about 350 nm (e.g., about 340 nm) and measuring the intensity of the second fluorescent emission having a wavelength of from about 380 nm to about 470 nm (e.g., 440 nm).

The ratio of the two intensities measured above can be calculated and compared to a control ratio. What is meant by control ratio is an established standard ratio (e.g., an established norm for the subject's age, sex, and/or race) or a ratio obtained from the subject (e.g., previously obtained from the same area of skin or obtained from another area skin such as an area of skin not readily exposed to UV radiation such as the underarm or buttocks). The method, thus, is able to determine the skin health of the subject (e.g., by comparing the ratio to the control ratio). The difference of the ratio value between exposed areas of skin and protected areas of skin has been found to generally decline with age. This difference is believed to be indicative of the ability of skin to react to external stimuli by repairing itself. Therefore, higher ratio values of the exposed areas compared to the unexposed areas is believed to be an indication of healthy skin, able to regenerate itself. Furthermore, high ratio values of the

exposed site compared to the unexposed site is also believed to be indicative of the youthfulness of the skin.

In one embodiment, the method is used to determine the effect of effect of a treatment to the skin of a subject. Examples of such treatments include, but are not limited to, cosmetic and pharmaceutical treatments (e.g, topical, parenteral, or oral), laser treatment, or abrasive treatment (e.g., microderm abrasion). In one embodiment, the treatment is a topical composition, such as a topical lotion or cream containing an anti-aging agent such as a retinoid (e.g., retinoic acid or retinol).

Applicants have found that while the fluorescence due to tryptophan moieties was found to decrease monotonically with age, the fluorescence bands assigned to pepsin and collagenase digestible collagen cross-links as well as that due to elastin cross-links was found to increase. These trends were surprisingly found to be independent of geographical region and seasonal effects. Similar trends were also observed in sun-protected areas. A marker that strongly correlates with skin aging, based on the ratio of the fluorescence intensity due to tryptophan moieties (centered at 295 nm excitation) to the fluorescence intensity assigned to collagen and elastin cross-links (centered at 390 nm excitation), was also found. This marker was found to decrease with aging, and photoaging was found to accelerate the rate of the decrease. Normalized tryptophan fluorescence was also shown to be able to be used to monitor the effects of anti-aging treatments.

Fluorescence Measurements

In vivo fluorescence spectroscopy can be performed for example using a fiber optic probe attached to a

spectrofluorimeter (e.g. the model SkinSkan (JY Horiba, Edison, NJ)). The method requires: a) a UV radiation source (e.g., a Xenon arc lamp or a mercury lamp), b) a method of selecting the radiation wavelength (e.g. a monochromator, a prism, or a grating), c) a method of delivery of the radiation to the tissue (e.g. a fiber bundle), d) a method of collection of the emitted radiation from the tissue (e.g. a fiber bundle), e) a method of selecting the emitted radiation wavelength (e.g. a monochromator, a prism, or a grating), and f) a method of detecting the emitted radiation (e.g. a photomultiplier, a single photodiode, a photodiode array, or a CCD array). See, e.g., Stamatas GN, et al., *J Invest Dermatol* 118(2):295-302, 2002.

Measurements were preformed by placing the fiber optic probe in contact with the skin site of interest. Before each set of measurements, the instrument was spectrally calibrated for excitation and emission in the region 250-650 nm. The chromatic resolution of the spectrofluorimeter was ±1 nm.

Acquisition of excitation spectra was the preferred method of measuring *in vivo* skin fluorescence. The reason for this choice over acquisition of emission spectra was that excitation spectra were similar to absorption spectra and bands tend to be narrower than in emission acquisition, both of which help in the identification of the individual fluorophores in a complex spectrum. The excitation spectra that were used in this study were the following: a) excitation scanned from 240 nm to 320 nm with emission set at 340 nm (tryptophan excitation maximum at 295 nm), b) excitation scanned from 240 nm to 380 nm with emission set

at 390 nm (pepsin digestible collagen cross-link excitation maximum at 335 nm), c) excitation scanned from 240 nm to 410 nm with emission at 420 nm (collagenase digestible collagen cross-link excitation maximum at 360 nm), d) excitation scanned from 260 nm to 490 nm with emission set at 500 nm (elastin cross-links - desmosine and isodesmosine - excitation maximum at about 390 nm).

In order to account for variations in skin native pigmentation that attenuates the detected fluorescence signal, fluorescence intensity was normalized with the diffuse reflectance signal of the same skin site at the corresponding wavelength. See, e.g., Stamatas GN, et al., *J Invest Dermatol* 118(2):295-302, 2002. A diffuse reflectance spectrum can be acquired by synchronizing the excitation and emission monochromators to select the same wavelength, scanning the range from 240 nm to 500 nm. The correction was necessary especially for wavelengths greater than 315 nm. The measured fluorescence in this wavelength region arises from the dermis (Gillies *et al*, 2000, Kollias *et al*, 1998), which means that excitation light has to travel through the whole epidermis where it is attenuated by epidermal melanin and proteins, both of which absorb strongly in the UV. Then the emitted light has to travel again through the whole epidermis to the collection fibers. This means that both excitation and emission intensities are compromised. On the other hand, for fluorophores that reside in the epidermis, i.e. signals for excitation wavelengths less than 315 nm (Gillies R, et al., *J Invest Dermatol* 115(4):704-707, 2000), the attenuation effect is not as severe. Furthermore, the intensity of the light source is low below 300 nm and normalization of the fluorescence by the diffuse reflectance signal in this

wavelength range amplified the noise. This problem arises only for the tryptophan band (295 nm excitation). To overcome this problem, the tryptophan fluorescence signal may be normalized to another fluorescence band, rather than to the diffuse reflectance value at 295 nm. Normalizing the tryptophan band to the 390 nm excitation band was used since the latter was found to change the slowest with aging. Other bands can also be used for the normalization. Also if the radiation source intensity is sufficient at about 295 nm normalization with the diffuse reflectance signal at this wavelength can be used.

Clinical Studies

To study the effects of aging on the native fluorescence of human facial skin, spectra was acquired from the cheek area of 522 healthy individuals with ages 15-75 years native to five different geographical locations of the Asia - Pacific region: a) Guangzhou, China, b) Harbin, China, c) Shanghai, China, d) Sendai, Japan, and e) Manila, Philippines. In order to identify potential seasonal effects, facial fluorescence from the same individuals was measured in summer and in winter at two locations (Harbin and Shanghai). All subjects were of skin type II-IV.

To investigate whether the observed changes on facial skin fluorescence were due to chronological aging or sun exposure (photoaging), a second set of measurements were conducted. Skin fluorescence was measured on the upper inner arm ("unexposed site") as well as the cheek area of 45 healthy subjects with ages 22-63 years. All subjects

were of skin type II-IV. The study was conducted in Skillman, New Jersey in the month of October.

In a third set of experiments, the effect of retinol on facial skin fluorescence was studied. Twenty healthy individuals of Caucasian decent (skin types II-III) with ages 50-70 years were asked to apply a cream formulation containing 0.15 % retinol containing a broad band spectrum SPF 15 sunscreen on one side of the face and a matched SPF 15 vehicle control (no retinol) on the other side daily. Active and vehicle were randomly assigned to each side of the face of every subject. The participants of the study, as well as the investigators, were blind to the assignment code. The study was carried out in Tucson, Arizona and commenced in February. Fluorescence excitation spectra were acquired at baseline, at three months, and at six months from both cheeks and from the upper inner arm as untreated sun-protected control. Diabetic patients were excluded as this condition may interfere with the fluorescence measurements.

Data Analysis

Linear regressions of the data were calculated using the least square errors algorithm. The goodness of fit is given by the correlation coefficient (R^2). Statistical significance was calculated using the Student's t-test for paired data distributions.

Results

The intensity of skin fluorescence was found to change with age. A series of typical excitation spectra taken on the cheek area of two individuals of 30 and 60 years of age, both of skin type II is shown in Fig. 1. In general,

the fluorescence excitation band ascribed to the tryptophan moieties (295 nm) decreases with age, whereas the bands of collagen and elastin cross-links (335 nm, 360 nm, and 390 nm) increase.

The age distribution of the fluorescence intensity for the 295 nm, 335 nm, 360 nm, and 390 nm excitation bands taken from 108 individuals in Shanghai, China is shown in Figs. 2a, 2b, 2c, and 2d correspondingly. The data has been fitted with linear regressions and the intervals between the average ± one standard deviation are shown. It is evident that the value of the standard deviation of the data distribution was higher for younger ages for the 295 nm excitation band. The opposite was found for all the other bands. The 295 nm excitation band was the only one declining with age (at -0.002 units/year). All the bands ascribed to collagen or elastin cross-links increased, indicating accumulation of extracellular matrix cross-links with age. From these bands, the 390 nm band showed the slowest increase with age (0.005 units/year). The correlation coefficient (R^2) was best for the 390 nm band (0.61) followed by the bands 360 nm (0.55), 335 nm (0.41), and 295 nm (0.32).

The same trends were observed independent of geographical area, skin type, or season of measurements. The slopes of the best linear fit of the data represent the rates of change of the fluorescence intensities and are shown in Table I. Rates of change (units/year) for the skin fluorescence bands and the normalized tryptophan fluorescence (I_{295nm} / I_{390nm}). All measurements were preformed on the face (cheek). The rates of change were calculated from the slopes of the best linear fit of the data. The

values are given in fluorescence units per year for the fluorescence bands and in ratio units per year for the normalized tryptophan fluorescence. PDCXL = pepsin digestible collagen cross-links, CDCXL = collagenase digestible collagen cross-links, NTF = normalized tryptophan fluorescence.

Table I

Geographical Area	Season	n	Tryptophan 295 nm	PDCXL 335 nm	CDCXL 360 nm	Elastin 390 nm	NTF Ratio 295 nm/390 nm
Guangzhou, China	Summer	108	-0.0021	0.010	0.013	0.0053	-0.103
Harbin, China	Summer	106	-0.0007	0.012	0.015	0.0062	-0.074
Harbin, China	Winter	64	-0.0025	0.014	0.016	0.0047	-0.091
Shanghai, China	Summer	100	-0.0017	0.012	0.018	0.0060	-0.119
Shanghai, China	Winter	100	-0.0024	0.012	0.013	0.0053	-0.135
Sendai, Japan	Summer	108	-0.0019	0.010	0.016	0.0047	-0.128
Manila, Philippines	Summer	100	-0.0003	0.002	0.010	0.0060	-0.038
Skillman, NJ, USA	Fall	45	-0.0773	0.008	0.006	0.0063	-0.088

In all geographic regions where the study took place and independent of the season, the fluorescence intensity of the tryptophan band decreased with age, while the intensities of the other three bands increased. Furthermore, the values of the slopes were fairly close together within the limits of uncertainty.

Normalizing the fluorescence intensity value of the 295 nm band to any of the other three bands resulted in a fluorescence marker that was relatively independent of skin

pigmentation. Furthermore, since the intensity of the 295 nm band decreased, while the intensities of the bands ascribed to cross-links increased with age, the above mentioned ratio resulted in stronger age dependence. The ratio that resulted in the strongest age dependence was that of the fluorescence intensity of the 295 nm band (I_{295nm}) over the fluorescence intensity of the 390 nm band (I_{390nm}). The age distribution of the normalized tryptophan fluorescence (I_{295nm} / I_{390nm}) for Shanghai, China is shown in Fig. 3. In all these figures the data has been fitted with linear regressions and the intervals between the average ± one standard deviation are provided. The standard deviation of the data distribution was higher for younger ages, however there was no significant correlation of the coefficient of variance (mean / standard deviation) with age. The correlation coefficient values were 0.4-0.5 for all places with the exception of Manila (R^2 = 0.15).

The cheek area was selected as it was expected to have received solar UV radiation that results in cumulative skin damage over a lifetime. To investigate whether sun exposure (photoaging) affected the observed decrease of the normalized tryptophan fluorescence with age, measurements were performed on the upper inner arm (relatively unexposed site) as well as on the cheek (sun-exposed site) of 45 volunteers. The results are shown in Figs. 4a and 4b. In accordance with the data presented in Fig. 3, the fluorescence ratio I_{295nm} / I_{390nm} acquired from the face decreased with age (Fig. 4a). The rate of decrease (0.087 units/year) was close to the values from other regions noted in Table I. For the sun-protected site the normalized tryptophan fluorescence was also decreasing with

age (Fig. 4b), although at a much slower rate (0.010 units/year).

In vivo skin fluorescence measurements was used to follow the anti-aging effects of topical treatment with retinol. The results for the cheek sites treated with the formulation containing 0.15 % retinol or the vehicle formulation are shown in Figs. 5a, 5b, and 5c. Both groups demonstrated a typical decrease in the 295 nm band fluorescence (Fig. 5a), although the rate of decrease was significantly less for the cheeks that received retinol treatment (-0.01 units/month for the active treated group versus -0.04 units/month for the placebo treated group). The 390 nm band did not significantly change over the period of the study, although a slight increasing trend was evident in both active and placebo treated groups (Fig. 5b). Normalization of the tryptophan fluorescence band to the 390 nm excitation band (Fig. 5c) showed that the decrease of the 295 nm band in the retinol treated group was most likely due to pigmentation increase in the subjects over the period of the study (note that the study took place between February and July). The rate of change in the normalized tryptophan fluorescence values in the vehicle treated group was -0.062 ± 0.029 ratio units per month. For the sites receiving retinol treatment, the decrease of the intensity of the 295 nm band was attenuated significantly compared to vehicle treated sites ($p < 0.01$). The normalized tryptophan fluorescence values remained virtually constant for the retinol treated sites, significantly different ($p < 0.05$) from the corresponding values for the sites that received vehicle treatment.

Measurements acquired on the upper inner arm (untreated) of the individuals at times 0, 3, and 6 months of the study demonstrated that the normalized tryptophan fluorescence values were decreasing, although at a much slower rate than the placebo treated skin in accordance with the data shown in Fig. 4b. The anti-aging effects of retinol treatment measured with fluorescence were in agreement with visual observations of reduction in the appearance of wrinkles in the treated areas. Treatment with vehicle cream alone did not have an effect on the appearance of wrinkles.

It is understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the claims.

What is claimed is: